SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

BE IT KNOWN THAT I, Dr. Jong Y. Lee, a citizen of the U.S. and a resident of Minneapolis, Hennepin County, Minnesota, have invented certain new and useful improvements in

PURIFIED HUMAN ERYTHROPOIETIN RECEPTOR PROTEIN FRAGMENT AND ANTIBODIES DERIVED THEREFROM

of which the following is a specification.

PURIFIED HUMAN ERYTHROPOIETIN RECEPTOR PROTEIN FRAGMENT AND ANTIBODIES DERIVED THEREFROM

Field of the Invention

This invention relates to purified human erythropoietin receptor extracellular domain polypeptide. More particularly, this invention relates to human erythropoietin receptor extracellular domain polypeptide that retains affinity for erythropoietin, to DNA sequences suitable for use in producing such a polypeptide, and to antibodies recognizing such a polypeptide.

Background of the Invention

Erythropoietin (Epo) is a glycoprotein hormone of molecular weight 34 kilodaltons (kDa) that is produced in the mammalian kidney and liver. Epo is a key component in erythropoiesis, inducing the proliferation and differentiation of red cell progenitors. Epo activity also is associated with the activation of a number of erythroid-specific genes, including globin and carbonic anhydrase. Bondurant et al., Mol. Cell Biol. 5:675-683 (1985); Koury et al., J. Cell. Physiol. 126:259-265 (1986). The erythropoietin receptor (EpoR) is a member of the hematopoietic/cytokine/growth factor receptor family, which includes several other growth factor receptors, such as the interleukin (IL)-3, -4 and -6 receptors, the granulocyte macrophage colony-stimulating factor (GM-CSF) receptor as well as the prolactin and growth hormone receptors.

Bazan, Proc. Natl. Acad. Sci USA 87:6934-6938 (1990). Members of the cytokine receptor family contain four conserved residues and a tryptophan-serine-X-tryptophan-serine motif positioned just outside the transmembrane region. The conserved sequences are thought to be involved in protein-protein interactions. Chiba et al., Biochim. Biophys. Res. Comm. 184:485-490 (1992).

EpoR cDNA has been isolated recently from mouse liver, Tojo et al., Biochem. Biophys. Res. Comm. 148: 443-48 (1987) and from human fetal liver. Jones et al., Blood 76:31-35 (1990); Winkelmann et al., Blood 76:24-30 (1990). The human cDNA encodes a polypeptide chain of MW ~55 kDa and having about 508 amino acids. Genomic clones of human EpoR have been isolated and sequenced. Penny and Forget, Genomics 11:974-80 (1991); Noguchi et al., Blood 78:2548-2556 (1991). Analysis of the coding sequence predicts about 24 amino acid residues in a signal peptide, about 226 amino acids in an extracellular domain, about 23 amino acids in a membrane-spanning domain, and about 235 amino acids in a cytoplasmic domain. D'Andrea and Zon, J. Clin. Invest. 86:681-687 (1990); Jones et al., Blood 76:31-35, (1990); Penny and Forget, Genomics 11: 974-80 (1991). The mature human EpoR protein has about 484 amino acids. All human erythroid progenitor cells have been shown to contain Epo receptors. Binding of Epo appears to decline as erythroid progenitor cells mature, until Epo receptors are not detectable on reticulocytes. Sawada et al., J. Clin. Invest. 80:357-366 (1987). Sawada et al., J. Cell. Physiol. 137:337 (1988). Epo maintains the cellular viability of the erythroid progenitor cells and allows them to proceed with mitosis and differentiation. Two major erythroid progenitors responsive to Epo are the Burst-

forming units-erythroid (BFU-E) and the Colony-forming units-erythroid (CFU-E). The Epo receptor number correlates very well with the response to Epo in normal BFU-E and CFU-E. Epo receptor numbers appear to decline after reaching the peak receptor number at the CFU-E stage in human and murine cells. Sawada et al., J. Clin. Invest. 80:357-366 (1987); Landschulz et al., Blood 73:1476-1486 (1989). The recovery of Epo receptors after removal of Epo appears to be dependent on protein synthesis, which suggests downregulation of Epo receptor by degradation, and the subsequent upregulation of receptors by the new synthesis of receptors when Epo is removed. Sawyer and Hankins, Blood 72:132 (1988). Studies of Epo receptors on megakaryocytes and erythroid progenitors suggest that there is a link between the regulation of erythropoiesis and thrombopoiesis, in that stimulation of cell division by both cell types is controlled by Epo receptor numbers. Berridge et al., Blood 72:970-977 (1988). Although the Epo receptor has been cloned, the precise mechanisms involved in binding of Epo to Epo receptors and the relationship to subsequent erythropoietic processes are not known.

Characterization of the Epo receptor (EpoR) has been difficult due to the extremely small quantities of EpoR that can be obtained from natural sources. Thus, the mechanism of Epo interaction with its receptor, which stimulates erythropoiesis, is still unknown. D'Andrea and Zon, J. Clin. Invest. 86:681-687 (1990). Recently this mechanism has been of great interest in understanding the role of growth factors and their receptors in leukemogenesis; altered hematopoietic growth factors and their

receptors may contribute to tumorigenesis and leukemogenesis. Dunbar et al., Science 245:1493-1496 (1989); Li et al., J. Virol. 57:534-538 (1986).

Several studies of the correlation between the Epo responsiveness of a particular cell type and the affinity of the cell type for Epo have reported discordant results. These studies have used recombinant Epo or EpoR possessing some non-native amino acid sequence from the corresponding plasmid vectors. Berridge et al., Blood 72:970-977 (1988); Harris et al., J. Biol. Chem. 267: 15205-09 (1992). It is possible that tertiary structural changes and/or other features of these recombinant Epo or EpoR molecules have changed the characteristics of the native protein. Thus, it would be a significant advance to obtain substantially pure fragments of the Epo receptor, free of extraneous (e.g, vector) amino acid sequence. Although it could not be predicted whether or not such fragments would retain functional activity, nevertheless a purified extracellular domain fragment would be particularly useful since Epo binds to the extracellular domain of the Epo receptor.

Summary of the Invention

An expression vector is disclosed, comprising a first nucleotide sequence capable of expressing a polypeptide that has a thrombin proteolytic cleavage site near the carboxyl terminus and a second nucleotide sequence consisting essentially of nucleotides 73 to 750 of a full length human erythropoietin receptor cDNA coding sequence. The Epo receptor cDNA coding sequence fragment is positioned 3′ to (downstream of) the proteolytic cleavage site and is in the same translational reading

frame as the proteolytic cleavage site. The Epo receptor cDNA coding sequence fragment is oriented to be translationally contiguous with the first polynucleotide sequence.

A purified fusion protein is disclosed, comprising a first segment consisting essentially of a polypeptide produced by an expression vector and having a thrombin proteolytic cleavage site, and a second segment consisting essentially of about amino acid 25 to about amino acid 250 of the full length human erythropoietin receptor protein. The second segment is covalently coupled to the carboxyl end of the first segment. A purified protein, consisting essentially of about amino acid 25 to about amino acid 250 of the full length human erythropoietin receptor protein sequence, may be produced by thrombin cleavage of the fusion protein.

An antibody having affinity for a purified human erythropoietin receptor polypeptide extracellular domain is disclosed. The antibody has affinity for a polypeptide comprising about amino acid 25 to about amino acid 250 of the full length human erythropoietin receptor protein sequence.

An immunoassay composition comprising a solid phase reagent and the antibody operably coupled to the solid phase reagent, is disclosed. Also disclosed is an immunoassay composition comprising a solid phase reagent and the purified protein operably coupled to the solid phase reagent.

Methods for obtaining a substantially pure human erythropoietin receptor polypeptide consisting essentially of about amino acid 25 to about amino acid 250 of the full length human erythropoietin receptor protein are disclosed. The substantially pure

human erythropoietin receptor polypeptide retains the ability to bind specifically to erythropoietin. The methods include treating the fusion protein with thrombin under conditions allowing cleavage of the polypeptide from the fusion protein, to form a digest mixture; adding the digest mixture to a solid phase reagent having erythropoietin coupled thereto, under conditions allowing binding of the polypeptide with the solid phase reagent, to form a polypeptide-solid phase composition; washing the polypeptide-solid phase composition to remove unbound material; and eluting the substantially pure human erythropoietin receptor polypeptide from the polypeptide-solid phase composition.

Brief Description of the Figures

Figure 1 is a diagrammatic representation of pJYL26, a plasmid having about 678 bp of the 5′ coding sequence of human erythropoietin receptor cDNA inserted into the expression vector pGEX-2T. Figure 1 also depicts the recombinant fusion protein, EpoRex-th, that is expressed from pJYL26.

Figure 2a shows the absorbance at 280 nanometers (A_{280}) of fractions collected from purification of an E. coli cell extract, expressing EpoRex-th, on a glutathione affinity column. Figure 2b shows the A_{280} of fractions containing Epo-bp collected as a result of erythiopoietin affinity chromatography of thrombin treated EpoRex-th.

Figure 3 is a photograph of a Coomassie blue stained polyacrylamide gel, showing the cleavage of EpoRex-th by thrombin.

Figure 4 is a Western blot, showing binding of sheep anti-Epo-bp antibody to Epo-bp.

Figure 5 shows the binding of various concentrations of human ^{125}I-Epo to Epo-bp, in the presence and absence of unlabeled Epo.

Figure 6 is a photograph of a Coomassie blue stained polyacrylamide gel, showing the polypeptide bands observed after trypsin digestion of Epo-bp.

Detailed Description of the Invention

Despite the availability of recombinant human Epo and full-length human Epo receptor cDNA clones, little is known about the interaction of Epo and Epo receptor, or the signal transducing mechanisms involved in proliferation and differentiation of erythroid progenitor cells.

Plasmid expression vectors permit expression of a protein from cloned coding sequences that have been inserted into the vector. Expression vectors generally have a selectable marker and a replication origin for selection and maintenance of the vector in a host cell, as well as inducible regulatory elements for inducing high level expression of a polypeptide suitable for fusing to an inserted gene. It is preferred that convenient restriction sites be engineered into the vector downstream from a proteolytic cleavage site sequence. A preferred polypeptide to be fused to the Epo coding sequence fragment is glutathione S-transferase, possessing a thrombin proteolytic cleavage site at the carboxyl terminus.

An expression vector for the invention disclosed herein expresses the EpoR extracellular domain as part of a fusion protein that can subsequently be cleaved to yield purified EpoR extracellular domain. The coding sequence for the EpoR extracellular domain may be engineered in any manner suitable for inserting the sequence in the appropriate reading frame in the expression vector. For example, a pair of polymerase chain reaction (PCR) primers may be synthesized, such that the first primer corresponds to the coding sequence at the 5′ end of the extracellular domain and the second primer is complementary to the coding sequence of the 3′ end of the extracellular domain. The primers preferably have convenient restriction enzyme sites flanking the portions of the primers corresponding to the ends of the desired target sequences. The primers are used to amplify the EpoR extracellular domain from a full length human EpoR cDNA template. The resulting PCR product is then cloned into an expression vector. It is preferable to synthesize PCR primers having different restriction sites at each end, rather than the same restriction site. The presence of different restriction sites at each end of the PCR product facilitates the insertion of the human EpoR coding sequence fragment in the sense orientation.

High level expression of a fusion protein having human erythropoietin receptor extracellular domain as part of the fusion protein is achieved by inducing expression from the recombinant plasmid expression vector in a host cell culture. A fusion protein is hereinafter referred to as EpoRex-th and a purified human erythropoietin receptor extracellular domain hereinafter is referred to as Epo-bp. A cell protein extract is preferably prepared from an expressing E. coli culture in any suitable

manner. EpoRex-th may be purifed from the extract as desired. For example, the extract may be passed over a column having the ability to bind the portion of the fusion protein upstream of the Epo-bp coding sequence. The fusion protein will bind to the column, while other proteins in the extract are eluted in column washes with a buffer that allows binding of fusion protein to the column matrix. EpoRex-th can be subsequently eluted in high purity by changing the buffer conditions.

Purification of Epo-bp may be accomplished by cleaving purified EpoRex-th using an appropriate cleavage method. For example, the cleavage site between the upstream polypeptide and Epo-bp may be sensitive to cyanogen bromide or, alternatively, may be sensitive to site-specific protease cleavage. In a preferred embodiment, a thrombin proteolytic cleavage site is engineered into the upstream polypeptide, but 5' to the convenient restriction cloning sites positioned at the carboxyl terminus of the upstream polypeptide coding sequence.

The cleaved Epo-bp polypeptide segment may be separated from the upstream polypeptide segment by purification techniques such as size exclusion chromatography, isoelectric focusing, or affinity chromatography. Furthermore, more than one purification technique may be used, if desired, to achieve the appropriate degree of purification. A preferred purification technique is affinity chromatography. For example, a protease-treated fusion protein mixture may be applied to a column having agarose beads coupled to Epo. The cleaved Epo-bp segment will bind to the Epo-agarose, while the upstream polypeptide segment will pass through the column. Epo-bp may then be eluted by lowering the pH of the liquid phase.

In an embodiment of the invention, the coding sequence for amino acids 25 through 250 of human EpoR (hEpoR) is cloned into pGEX-2T (Pharmacia, Mechanicsburg, PA). pGEX-2T has an IPTG inducible promoter operably linked to a coding sequence for glutathione S-transferase (GST). The 3' end of the GST coding sequence has a thrombin proteolytic cleavage site in the correct reading frame, as well as convenient cloning sites for inserting a coding sequence to be covalently coupled to GST.

A PCR product having amino acids 25 through 250 of hEpoR is made from a suitable DNA template, for example a full-length human EpoR cDNA. A PCR primer is sythesized having the 5' end of the extracellular domain coding sequence as well as a BamH1 site, and a PCR primer is synthesized having sequence complementary to the 3' end of the extracellular domain coding sequence as well as an EcoR1 site. The BamH1 site in pGEX-2T is positioned 5' to the EcoR1 site relative to the GST coding sequence. The PCR product is cloned into pGEX-2T, and a transformed E. coli colony having a plasmid of the expected size is identified.

A fusion protein having an amino terminal GST segment and a carboxy terminal EpoR extracellular domain segment is expressed in transformed E. coli by inducing transcription with IPTG. IPTG derepresses the lac promoter positioned upstream of the fusion protein coding sequence. After allowing expression for a period of time sufficient to accumulate an amount of the fusion protein, cells are lysed and a crude extract is made in any suitable manner. The crude extract mixture has the fusion

protein in addition to many other cellular proteins. The fusion protein, EpoRex-th, may be purified from the extract as desired.

In a preferred embodiment, EpoRex-th is passed over a column having agarose beads coupled to glutathione (GSH). GSH is a substrate for GST, and the GST segment of EpoRex-th will bind to the immobilized GSH with high affinity. Thus, the fusion protein becomes bound to the column, while virtually all other proteins in the extract will not bind. After washing, EpoRex-th may be eluted from the column by adding reduced GSH to the liquid phase.

In an embodiment of the invention, purified human erythropoietin receptor extracellular domain polypeptide may be made by digesting EpoRex-th with thrombin. The resulting digested mixture of GST and Epo-bp may then be applied to an Epo affinity column. The Epo-bp binds to its ligand, Epo, whereas GST passes through the column. Epo-bp may be eluted in purified form through use of an appropriate elution buffer, for example 0.1 M glycine, pH 3.0.

Antibodies to human erythropoietin receptor extracellular domain can be made by presentation of a purified preparation of such a polypeptide to the immune system of an animal. For example, purified Epo-bp may be injected subcutaneously, intramuscularly or intraperitoneally into animals such as rats, mice, rabbits, or sheep. Booster injections can be given at intervals, if desired. Circulating antibodies against Epo-bp are made by the immune system of the injected animal, and these antibodies can be collected from the blood, preferably from the serum. Anti-Epo-bp serum can be used to detect Epo-bp in various assay formats, such as Western blots, ELISA assays and

the like. Epo-bp to be detected may be from, for example, a purified preparation of Epo-bp, a bacterial or eukaryotic cell extract, a eukaryotic cell from an in vitro cell culture, a serum sample, or even a tissue or cell biopsy taken from an individual. Anti-Epo-bp antibodies are expected to recognize the extracellular domain of intact human EpoR as well as Epo-bp. Monoclonal antibodies directed against Epo-bp can be made by methods known in the art. D'Andrea et al., Blood 75: 874-80 (1990); Goldwasser et al., U.S. Patent No. 4,558,005; Harlow and Lane, Antibodies - Lab Manual, Cold Spring Harbor Laboratory, 1988.

Antibodies directed against Epo-bp preferably have a specific binding affinity for the EpoR extracellular domain. For example, serum from an animal injected with purified Epo-bp should provide detectable binding to Epo-bp in Western blots when 10 μg of purified Epo-bp are electrophoresed in a polyacrylamide gel and exposed to a 1:2000 dilution of the anti-Epo-bp serum.

The purified extracellular domain of EpoR disclosed herein is the first such pure human Epo receptor fragment (i.e., free of non-human or non-Epo receptor amino acid sequence) to be obtained. The experiments disclosed herein demonstrate that such a fragment retains the ability to specifically bind human Epo. The proteins and antibodies disclosed herein are useful for understanding the mechanisms of Epo - Epo receptor interaction. The purified Epo-bp of the present invention is also useful for investigating the structure of the Epo receptor and for identifying factors involved in regulating differentiation and proliferation mechanisms in erythroid progenitor cells. Moreover, the invention disclosed herein is useful for identifying and quantitating Epo

and Epo receptor, as well as in understanding hematopoietic malignancy and certain cardiovascular system disorders. That is, increased/decreased hematocrit and/or hemoglobin levels may affect blood pressure and cause other circulatory problems.

The invention will be further understood with reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limiting the true scope of the present invention as described in the claims.

EXAMPLE 1

Materials

Glutathione (GSH)-agarose, pGEX-2T expression vector and Sephadex G-50 were purchased from Pharmacia (Mechanicsburg, PA). PCR reagents were from Perkin-Elmer Cetus (Norwalk, CT) and Affigel 15 was from BioRad (Richmond, CA). Bacteriophage T4 DNA ligase, restriction enzymes and isopropylthio-β-D-galactoside (IPTG) were purchased from BRL Gibco (Gaithersburg, MD). Geneclean II was from Bio 101, La Jolla, CA. Nitrocellulose was from Schleicher & Schuell Co. (Keene, NH). Chemiluminescence (ECL) reagents and ^{125}I-Epo were from Amersham (Arlington Heights, IL) and unlabeled Epo was a gift of Chugai-Upjohn (Rosemont, IL). Phenylmethylsulfonylfluoride (PMSF), diisopropylfluorophosphate (DFP), thrombin, trypsin and Triton X-100, were from Sigma Chemical Company (St. Louis, MO). Biotinylated rabbit anti-sheep antibodies and avidin-horseradish peroxidase were from Pierce Co. (Rockford, IL). LAP37, a full-length human erythropoietin receptor (EpoR)

cDNA preparation, was provided by Dr. Bernard G. Forget, Yale University, New Haven, CT. All other chemicals were of reagent grade.

EXAMPLE 2

Construction of EpoR cDNA Recombinant Vector

A recombinant plasmid expression vector, pJYL26, was constructed from a PCR product having the human Epo receptor extracellular domain coding sequence and from the plasmid vector pGEX-2T. The construction of this plasmid is explained below.

PCR amplification was carried out using a full-length human EpoR cDNA, LAP37, as a template. The 5'-sense primer was 5'-TTGGATCCGCGCCCCCGCCTAAC-3'. This primer has a BamH1 linker sequence at the 5' end, followed by the coding sequence for amino acids 25 through 29 of the full length human EpoR protein. The 3'-antisense primer was 5'-TGAATTCGGGGTCCAGGTCGCT-3'. This primer has an EcoR1 linker followed by sequence complementary to the coding sequence for amino acids 226 through 222 of full length EpoR. Using a Perkin Elmer-Cetus PCR kit, PCR was carried out with 0.1 μg of LAP37 cDNA, 20 pM of each primer, 1.25 mM dNTP mixture (dGTP, dCTP, dTTP and dATP), 0.5 μl of Taq polymerase, and 10x buffer supplied in the PCR kit. Amplification was carried out by a PTC-100 Programmable Thermal Controller, (M.J. Research, Inc. Watertown, MA), with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for $1^1/_2$ min, repeated for 25 cycles.

The sizes of the PCR product (~600 bp) and pGEX-2T (~4.9 kb) were verified on 1% Seakem and 2% Nusieve agarose (FMC Bioproducts, Rockland, ME) gels running in 1x TA buffer (50x TA in 1 liter volume containing 242 g Tris-base and 57.1 ml acetic acid), with a Hae II standard. Both the PCR product and pGEX-2T were purified from gel slices by the Geneclean II method as described by the manufacturer (Bio 101, La Jolla, CA). Concentrations of the PCR product and pGEX-2T were estimated by absorbance readings at OD260. Both DNAs were then digested with BamH1 and EcoR1 for 4 hours at 37°C before ligation. The digested products were analyzed on 1% Seakem and 2% Nusieve agarose gels. Both the PCR product and pGEX-2T fragments were cut from the gel and purified again by the Geneclean II method.

The ligation was done in a mixture having 1 µg/µl each of PCR product and pGEX-2T. The mixture was incubated at 45°C for 5 minutes and chilled to 0°C. Then, in a 10 µl final volume, 1 µl each of 10x bacteriophage T4 DNA buffer and 10x bacteriophage DNA ligase, and 10 mM ATP were added. The whole mixture was then incubated at 16°C in a circulating water bath overnight. Productive ligation was verified by electrophoresis in a 1% agarose gel in 1x TA buffer running at 100 volts with lanes containing size standards, pGEX-2T, PCR product, and the ligated product (PCR product + pGEX-2T). The ligated product was verified to be ~5.5 kb. An aliquot of ligation mixture was then transformed into E. coli strain JM109 (20 µg ligation mixture/200 µl JM109). For the transformation, the E. coli mixture was incubated on ice for 30 minutes after mixing gently by inverting, and incubated at 42°C exactly 90 seconds. Then the mixture was chilled on ice for 1-2 minutes and 500 µl LB medium

(for 1 liter, 10 g bacto-tryptone, 5 g bacto-yeast and 10 g NaCl , pH 7.5, autoclave) was added. After incubating at 37°C for 45 minutes, the LB mixtures were spread on LB/Amp agar petri plates in amounts of 50, 75, 125, 150,and 300 ml of LB mixture. Agar petri plates were prepared with 20-30 ml of LB/Amp medium, containing 15 g agar/liter LB (autoclaved) and 100 µg/liter ampicillin. Control LB/Amp plates were made with intact pGEX-2T, digested pGEX-2T and PCR product only. The plates were kept on the bench top to absorb liquid for a few hours and inverted plates were incubated at 37°C for 24 hours. Grown colonies were seeded on gridded plates, which were incubated again at 37°C for 24 hours, while another set of all colonies was grown in 5 ml each of the LB/Amp medium overnight.

The DNA was extracted from each colony by the miniprep method. Each colony was cultured overnight with 5 ml LB/Amp medium (2 µl/ml of 50 µg/ml Amp stock) in a loosely capped 15-ml plastic tube in a vigorously shaking 37°C incubator. The following day, 1.5 ml of each culture was pelleted in a microfuge for 3 minutes at 4°C at 14,000 x g, and resuspended in 93 µl STET plus 17 µl of lysozyme stock (STET: 5% sucrose + 5% Triton X-100 + 50 mM Tris, pH 8.0 + 50 mM EDTA, pH 8.0, stored at 4°C; lysozyme stock: 5 mg/ml, stored in a freezer). The resuspended mixture was then incubated for 10 minutes at room temperature and boiled for 2 minutes before spinning in a microfuge at 4°C for 15 minutes at 14,000 x g. The pellet was removed with a sterile tooth pick, 2 µl of RNAse (100 mg/ml) was added to the supernatant, followed by incubation at 37°C for 30 minutes. After incubation, 110 µl of ice-cold isopropanol was added and the mixture was inverted 4 times before pelleting at 14,000 x g, 4°C for 15

minute. The pellet (DNA) was then washed with ~1 ml of 70% ethanol to remove residual STET and other contaminants, and the pellet centrifuged again at 14,000 x g, 4°C for 15 minutes. The pellet was then air dried for 1-2 hours and resuspended in 25 µl of sterile dH_2O.

The extracted DNAs were verified on a 0.8% agarose gel in TA buffer, running at 100 volts until the front dye line migrated 4/5 of the length of the gel. The gel was stained with ethidium bromide (0.5 µg/ml) at room temperature for 15 minutes on a gentle shaker and destained with dH_20 for 15 minutes. DNA bands were examined under UV light. Cultures having DNA of the expected size were examined in 1% agarose gels running in TA buffer after EcoR1 and/or EcoR1 plus BamH1 digestion. The EcoR1 and BamH1 digestion was done by incubating the sample mixture at 37°C water bath for 2 hours with the mixture of 1 µg of EcoR1 or BamH1 per 2 µg of DNA in 1 µl/10 µl sample volume of 10x reaction buffer provided in the restriction enzyme kit. One colony having a plasmid of about ~5.5 kb in size was selected after examining both EcoR1 and EcoR1 plus BamH1 digested DNA sizes in 1% agarose gels. The plasmid in this colony was named pJYL26. A diagram of pJYL26 is shown in the upper part of Figure 1.

EXAMPLE 3

Purification of EpoRex-th Fusion Protein

This example teaches the production and purification of a fusion protein having two segments. The first segment is a polypeptide, GST, with a thrombin

cleavage site at the carboxyl terminus. The second segment, fused to the first segment at the thrombin cleavage site, is the extracellular domain of human Epo receptor. The fusion protein EpoRex-th, containing GST and Epo-bp, is purified by GSH-agarose affinity chromatography.

Transformed E. coli containing the recombinant vector pJYL26 were grown overnight at 37°C with vigorous shaking in 400 ml of LB medium with 100 µg/ml of ampicillin. The following day, the culture was diluted in 4 liters of fresh LB/Amp media and incubated for another 90 min before adding 1 mM isopropylthio-β-D-galactoside (IPTG). After 4 hours of IPTG induction, the cells were pelleted at 3,000 x g at 4°C for 15 min and resuspended in 160 ml of lysis buffer, containing 50 mM sodium phosphate, pH 7.4, 10 mM ß-mercaptoethanol (ßME), 10 mM EDTA, pH 8.0, 1 mM PMSF and 1 mM DFP. 160 mg of solid lysozyme was then added. Using a 60 cc syringe, the lysed cell suspension was homogenized by passing through 18, 21 and 23 gauge needles three times, and incubated on ice 30 min. After dry ice/methanol freeze thaw at 37°C for 3 times and mild sonication, 1% of Triton X-100 was added. The supernatant was collected by centrifugation 15 x kg at 4°C for 15 min.

A GSH-agarose column was prepared by washing swollen GSH-agarose beads 3 times with 10 bed volumes of phosphate-buffered saline (PBS: 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4 in excess salt of 3 M NaCl) to remove preservatives and elutable dextran from the agarose. The column was then equilibrated with 5 bed volumes of isotonic PBS. The IPTG induced extract was applied to the column and the column was washed twice with 5 bed volumes of PBS, which elutes all proteins with no affinity for

GSH-agarose. EpoRex-th was then eluted by adding 5 bed volumes of elution buffer, containing 5 mM reduced GSH in 50 mM Tris-HCl, pH 8.0. Fractions of 1.0 ml were collected and the A_{280} was determined for each fraction. Figure 2a shows the A_{280} data. Fractions 18-23 were subsequently shown to have the EpoRex-th protein. These fractions were pooled. From a four-liter cell culture preparation, an average of 2 mg of EpoRex-th was extracted.

EXAMPLE 4

Purification of Epo-bp

EpoRex-th contains a thrombin-specific proteolytic cleavage site, as diagrammed in the lower half of Figure 1. Thrombin cleaves specifically at the sequence -CTG GTT CCG CGT GGA TCC-, which codes for the amino acids Leu Val Pro Arg Gly Ser, as shown in Figure 1. Smith and Johnson, Gene 67:31-40 (1988). Thrombin was incubated with EpoRex-th to cleave the GST segment from the Epo-bp segment and the two segments were purified by Epo-agarose affinity, as described below.

Various thrombin concentrations were tested in order to find the most effective range of thrombin cleavage. Purified EpoRex-th was incubated with 0.0125, 0.125, 0.6 or 2.4 µg of thrombin per 60 µg EpoRex-th at room temperature or 37°C for 1 hour in PBS buffer, pH 7.4. The results were analyzed by polyacrylamide gel (12.5%) electrophoresis. After staining with Coomassie blue, bands could be seen corresponding to the fusion protein EpoRex-th (55 kDa), Epo-bp (29 kDa) and GST (26

kDa). The 0.6 µg concentration was selected for complete digestion of EpoRex-th. The results are presented in Figures 3.

For thrombin cleavage, 60 µg of EpoRex-th was incubated at room temperature for 1 hr with 0.6 µg thrombin. The mixture was applied to an erythropoietin - agarose column in Tris buffered saline (TBS) or PBS. Epo-bp was eluted with 0.1 M glycine buffer, pH 3.0. Fractions of 0.5 ml were collected into tubes, containing 0.5 ml of 2 M Tris-HCl, pH 7.5. Epo-bp peak fractions 14-19 were pooled and then dialyzed overnight in TBS or PBS at 4°C for further experiments. Approximately 200 µg Epo-bp was extracted, starting from a four-liter cell culture preparation.

The Epo-agarose column was prepared from Epo-agarose beads. The Epo-agarose beads were prepared by overnight dialysis of Epo (0.5 mg/ml) in 0.1 M 3(N-morpholino)-propanesulfonic acid (MOPS) at 4°C. Epo was linked to Affigel 15 beads by admixing 1 ml of the dialyzed Epo-solution and 2 ml of washed Affigel 15, and incubated at room temperature for 2 hours on a rotating shaker. The supernatant was removed after microcentrifuging at 2000 x g for 30 sec. The packed Epo-agarose beads were washed 3 times in TBS or PBS at 4°C and stored until ready to use. After collecting desired protein fractions, Epo-agarose beads may be washed extensively with TBS or PBS and stored at 4°C for reuse.

EXAMPLE 5

Production of Antibodies to Epo-bp

This example teaches the production of antibodies directed against purified Epo-bp. Purified Epo-bp is electrophoresed in a 12.5% SDS-PAGE gel and the Epo-bp protein band is resuspended in PBS and injected into sheep. Sheep serum having anti-Epo-bp antibody is shown to detect purified human Epo-bp when the serum is diluted 1:2000.

Epo-bp (0.5 mg), purified as described above, was mixed with 2x treatment (Laemmli) buffer and boiled for 10 minutes. The mixture was applied to a 12.5% SDS gel and electrophoresed at 200 volts for 3-4 hours. The gel was stained with 0.125% Coomassie blue overnight, destained 1-2 hours with dH_2O, and the Epo-bp band cut out of the gel with a razor blade.

The Epo-bp gel slice was resuspended in 10-15 ml of PBS buffer and passed through a syringe repeatedly until the gel was crushed into small pieces forming a suspension mixture with PBS. The suspension was injected subcutaneously in adult sheep. Epo-bp was injected at a ratio of 0.5 mg Epo-bp or more per 25 kg weight of the animal. Two booster injections, with the same dose as in the initial injection, were given once every 3 weeks following initial injection. After the second booster injection, blood can be withdrawn for collection of antibodies. Injections can be given every month to maintain antibody production by the animal. Injection sites are rotated on the animal. Sambrook et al., Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press, Chapter 18, 1989.

To obtain blood from injected animals, hair at the blood sampling site was cleaned with 70% alcohol. Ear arteries or other accessible arteries were shaved over. A small amount of xylene was applied to the tip of the ear but not at the bleeding site. Blood was gently withdrawn with a butterfly and put into a glass tube having no heparin. The blood was incubated at room temperature for 1 hour to allow clotting, the clot was loosened from the tube wall with a pasteur pipet, and the tube was incubated at 4°C overnight. The clotted blood mixture was poured into a dish and the clot removed. The unclotted remainder was returned to the glass tube and centrifuged at 3000 rpm for 10 minutes. The supernatant (serum) was applied to an Epo-bp-affinity column and antibodies binding to the column were eluted by with 0.1 M glycine buffer, pH 3.0, using the same procedures as discussed above for purification of Epo-bp. The eluate was dialyzed in PBS overnight at 4°C and stored at -70°C in 500 µl aliquots. The Epo-bp affinity column was prepared from Epo-bp and Affigel 15 agarose beads in the same manner as the Epo-bp Affigel beads described in Example 6 below.

Solutions used in this example are prepared as follows:

Lysis Buffer II: 50 mM $NaP0_4$ (7.74 ml of 0.5 M dibasic PO_4 plus 2.26 of 0.5 M monobasic PO_4) + 10 mM β-mercaptoethanol + 10 mM EDTA, pH 8.

PBS Buffer: 0.15 M NaCl + 16 mM dibasic $P0_4$ + 6 mM monobasic $P0_4$, pH 7.4.

TBS buffer: for 1 liter, 12.5 ml of 2 M Tris-HCl, pH 7.4 + 27.5 ml of 5 M NaCl.

2x Treatment (Laemmli) buffer: 0.125 M Tris-HCl, pH 6.8 + 4% SDS + 20% glycerol + 10% beta-mercaptoethanol.

Sheep anti-Epo-bp serum was analyzed for binding to purified Epo-bp by Western blotting as described in Sambrook et al., Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989 and in Western blotting protocols provided by the ECL manufacturer, Amersham Co., Arlington Heights, Il. Following thrombin cleavage, EpoRex-th and Epo-bp were separated electrophoretically on an SDS-PAGE gel. The gel was then blotted onto nitrocellulose (Schleicher and Schuell Co., Keene, NH). Sheep anti-Epo-bp serum was added to the nitrocellulose in Blotto (for 1 liter: 80 g non-fat dry milk, 30 ml 5M NaCl, 10 ml 2M Tris-HCl, pH 7.5 and 0.05% Tween-20) at a 1:2000 dilution and incubated at room temperature for 1 hour with gentle agitation. After rinsing off the first antibody, a second reagent, biotinylated rabbit anti-immunoglobulin anti-sheep (1:10,000 dilution) antibody was added to the nitrocellulose in Blotto, and incubated at room temperature for another 1 hour with rocking. Horseradish peroxidase-avidin (1:10,000 dilution) was added and the mixture incubated at room temperature for 45 min. After soaking the washed nitrocellulose briefly in chemiluminescence (ECL) reagents, wet blots were exposed immediately on KODAK X-ray film. Figure 4 shows a photograph of the Western blot, with the lanes having the following proteins applied: Lane 1, molecular weight standards; Lane 2, thrombin digested EpoRex-th; Lane 3, GST; Lane 4, purified Epo-bp. As shown in lane 4 of Figure 4, purified Epo-bp was detected by a 1:2000 dilution of anti-Epo-bp antibody. The apparent molecular weight of the purified Epo-bp was about 29 kDa.

EXAMPLE 6

Binding of Epo to Epo-bp

Ligand binding of Epo to Epo-bp and effects of Epo concentration on binding are taught in this example.

Epo-bp beads were prepared by adding 60 µg/ml Epo-bp to washed Affigel 15 agarose beads in PBS, with a final concentration of approximately 30 µg of protein per 1 ml of Epo-bp beads. The mixture was incubated at room temperature for 2 hours on a rotating platform. After washing 3 times with ice cold PBS buffer, the pellet was resuspended in 1 ml of PBS buffer. For binding assays, 30 ul of the final suspension (approximately 1.0 µg of Epo-bp) were admixed with various concentrations of ^{125}I-Epo and incubated for 1 hour at room temperature while resuspending every 5 min with a pipet. At the end of the incubation, 1 ml of ice cold PBS buffer was added to wash out unreacted ^{125}I-Epo and the wash was repeated twice more. The reacted beads were counted by a gamma counter. Proteins smaller than the intact Epo-bp from trypsin digested extracts (see below) were also applied in the same way to test any effect on ligand binding. Nonspecific binding was measured by the same method except the mixture was preincubated with a 200-fold excess of unlabeled Epo for 1 hour prior to adding labeled Epo.

Binding of Epo-bp to Epo is shown in Figure 5. Each point in Figure 5 is the mean of 2-4 samples. Data are expressed as mean ± SEM. A p value of less than 0.05 was considered significant. Results were analysed with the two-tailed Student t-test. The specific binding activity of Epo to Epo-bp dramatically increased as Epo

concentration increased; the binding tripled from 8 nM to 12 nM ^{125}I-Epo. Apparent saturation of Epo binding occurred at 12 nM. This was also confirmed in the unreacted supernatant of ^{125}I-Epo. Binding of ^{125}I-Epo to Epo-bp was significantly inhibited in the presence of unlabeled Epo at concentrations of 8 nM and higher of ^{125}I-Epo ($p < 0.0001$ in both comparisons). Nonspecific binding was somewhat higher than expected. It had been expected that the excess unlabeled Epo might eliminate ^{125}I-Epo binding completely because of the sensitivity and specificity of Epo binding to Epo-bp shown in Western blots and binding assays.

Trypsin digestion experiments were performed to find a minimum sequence of Epo-bp involved in ligand binding. There are several arginine and lysine sites in the Epo receptor protein, which may be specific sites for trypsin digestion. Trypsin digestion of Epo-bp was carried out at 10, 20, 30, 50, 100 µg and 2 mg of trypsin per 5 µg of Epo-bp in a total volume of 200 µl in PBS, pH 6.7 at 37°C for 3 or 6 hours. The reaction was stopped by adding the same volume of 2 N acetic acid or by boiling. As shown in Figure 6, Epo-bp was cleaved effectively when 20 µg or more of trypsin was present. Trypsin is visible as a 23.2 kDa protein band in the lane having 2 mg of trypsin. The trypsin digested Epo-bp is visible as a 20-kDa protein. In Figure 6, Lane 1 contains standard molecular weight markers; lane 2 is a control; lanes 3-8 represent digestions at concentrations of 10, 20 30, 50, 100 µg and 2 mg trypsin, respectively at 37°C for 3 hours; lanes 9-14 represent the same concentrations of trypsin incubated at 37°C for 6 hours.

Since uncut Epo-bp is aproximately 30 kDa, gel filtration chromatography using Pharmacia Sephadex G-50 (MW ≤ 30,000) was applied to separate protein components of size ≤ 30,000 molecular weight from the total mixture. A powdered form of Sephadex G-50 was hydrated and washed several times with isotonic PBS to wash out preservatives. Trypsin digested EpoRex-th was applied to the top of the gel column in a total volume of 0.2 ml in PBS. The column was centrifuged at 2,000 x g for 4 min at room temperature in a swinging-bucket rotor. The first effluent was collected from the bottom of the syringe (~0.2 ml) into a decapped microfuge tube. This effluent contains proteins having a size larger than Epo-bp. Another 0.2 ml of PBS buffer was added to the column and a second eluate collected into a new decapped microfuge by recentrifuging for 10 min. This step was repeated twice. The second eluate was applied to an Epo-agarose column and peak fractions were examined by SDS-PAGE gels and Western blotting. The final product of Epo-bp, as a result of trypsin digestion, was approximately 20 kDa, shown in Figure 6. The antibody did not recognize the cleaved Epo-bp. Thus, deletion of 30 amino acids from Epo-bp by trypsin digestion completely eliminated recognition by antibodies to Epo-bp, as verified by Western blotting.

The foregoing detailed description has been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the appended claims.